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Purification and properties of an *N*-acetylgalactosamine specific lectin from the plant pathogenic fungus *Rhizoctonia solani*

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A lectin was isolated from *Rhizoctonia solani* mycelium by affinity chromatography on gum arabic-Sepharose. It is a dimeric protein composed of 2 identical subunits of 13 kDa with high contents of asparagine/aspartic acid, valine, glycine, glutamine/glutamic acid and lysine. The *R. solani* agglutinin (RSA) exhibits specificity towards *N*-acetylgalactosamine, and preferentially agglutinates human type A over type B and type O erythrocytes.

Lectin; Purification; *N*-Acetylgalactosamine; Gum arabic; (*Rhizoctonia solani*)

1. INTRODUCTION

Many species of living organisms of almost every taxonomic classification ranging from bacteria to higher animals contain carbohydrate binding proteins known as agglutinins or lectins [1,2]. Although in the past, most of the research has been done on plant and animal lectins, evidence has accumulated during the last decades that many fungi contain agglutinating substances. Lectins have been isolated from fruiting bodies of several higher fungi including commercial mushroom (*Agaricus bisporus*) [3], stinkhorn (*Phallus impudicus*) [4], *Agrocybe aegerita* [5], *Laccaria amethystina* [6], *Pleurotus ostreacus* [7], *Clitocybe nebularis* [8], *Fomes fomentarius* [8] and *Flammulina velutipes* [9]. Lectins have also been found in lower fungi such as the parasitic *Phycomycete conidiobolus* [10] and the soil born plant pathogen *Rhizoctonia solani* [11]. The L-fucose binding agglutinin of the latter species was found on the surface of the hyphae [12] and is believed to play a role in the recognition and adherence of the mycoparasite *Trichoderma* [11]. This report deals with another *R. solani* lectin, which is a soluble intracellular-protein exhibiting specificity towards *N*-acetylgalactosamine and several other simple sugars.

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Abbreviations: PBS, phosphate-buffered saline; RSA, *Rhizoctonia solani* agglutinin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

All sugars are of the D-configuration, unless otherwise stated

2. MATERIALS AND METHODS

2.1. *Materials*

R. solani (strain 08103 isolated from tomato by F. Van Achter, KU Leuven, Faculty of Agronomy)

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was grown on a synthetic medium (SM) [13] in 3 l Erlemeyer flasks containing 1 l of SM on a rotary shaker (New Brunswick) at 200 rpm and 30°C. Mycelium of 15-day old cultures was collected on a Büchner funnel, washed with distilled water and air-dried (on the Büchner funnel under partial vacuum) in order to remove excess water. The partially dried mass was frozen at −80°C, lyophilized, frozen in liquid nitrogen and pulverized in a coffee mill. The resulting powder was either used immediately or stored at −20°C. Typical yields varied around 10 g dry powder per l culture.

2.2. *Preparation of immobilized gum arabic*

Soluble gum arabic was coupled to Epoxy-activated Sepharose-6B (Pharmacia, Uppsala, Sweden) according to the manufacturers instructions using a 10% solution of gum arabic (Merck, Darmstadt, FRG).

2.3. *Isolation of the* Rhizoctonia solani *agglutinin (RSA)*

Powdered mycelium was extracted in phosphate-buffered saline (PBS; 25 ml/g) on a magnetic stirrer. The resulting homogenate was centrifuged (15 min; 20000 × *g*), the supernatant taken off and the pellet reextracted with 25 ml/g of PBS. After centrifugation (15 min; 20000 × *g*) of the second extract, both supernatant fractions were pooled and applied (in portions of 100 ml per chromatography) to a column (10 ml bed volume) of immobilized gum arabic preequilibrated with PBS. Unbound proteins were eluted with PBS (until the A_{280} of the eluate fell below 0.01) and the lectin desorbed with 20 mM (unbuffered) 1,3-diaminopropane. Under these conditions virtually all lectin activity was retained on the column and eluted in a sharp peak (about 30 ml) upon raising the pH with diaminopropane. Alternatively, the lectin can be desorbed with 0.1 M galactose in PBS. However, since both desorbants yield equal amounts of RSA (in terms of agglutination units as determined by agglutination assays) of the same purity (determined by SDS-PAGE as shown in fig.1), elution of the lectin by increasing the pH was chosen for practical reasons (e.g. ease of regeneration of the column, absence of inhibitory saccharide in the affinity-purified RSA). To ensure complete purity of our RSA-preparation, affinity-purified fractions (desorbed with unbuffered diaminopropane) were dialysed against formate buffer (50 mM Na-formate, pH 4.0) and chromatographed on a Pharmacia Mono-S cation-exchange column using a fast protein liquid chromatography system (fig.2). Peak fractions were collected and used for further analyses.

2.4. *Assays and analytical methods*

Protein was estimated as in [14]. Agglutination assays were carried out with trypsin-treated rabbit erythrocytes as in [15] (unless stated otherwise). SDS-PAGE was done on a 12.5–25% polyacrylamide gradient gel using a discontinuous system as in [16]. Amino acid and carbohydrate analysis were carried out as in [17], the neutral sugars being analyzed by GLC and the amino sugars on the amino acid analyser. Tryptophan was determined spectrophotometrically.

3. RESULTS

3.1. *Purifications of RSA*

A preliminary experiment with crude extract from *R. solani* mycelium indicated that the lectin exhibits specificity towards *N*-acetylgalactosamine. However, RSA bound very poorly to immobilized *N*-acetylgalactosamine (e.g. Selectin 5 from Pierce, Rockford, IL, USA). In addition the lectin was not retained on other commonly used affinity supports such as fetuin-agarose, thyroglobulin-agarose and ovomucoid-agarose (not shown). Therefore, a matrix, based on immobilized gum arabic was employed, which allowed the isolation of RSA with a high efficiency. Up to 98% of the total lectin present in the crude extract was recovered in the affinity-purified RSA fraction. Under conditions of saturation, up to 5 mg lectin was bound per ml of affinity support. To remove possible contaminants an additional purification step based on ion-exchange chromatography was included in the purification scheme. As shown in fig.2, our final RSA preparations eluted in a single symmetrical peak from the Mono-S column which demonstrates that (i) the lectin is essentially pure and (ii) behaves as a single molecular species.

The overall yield of RSA was around 15 mg/g (lyophilized) mycelium, which corresponds to

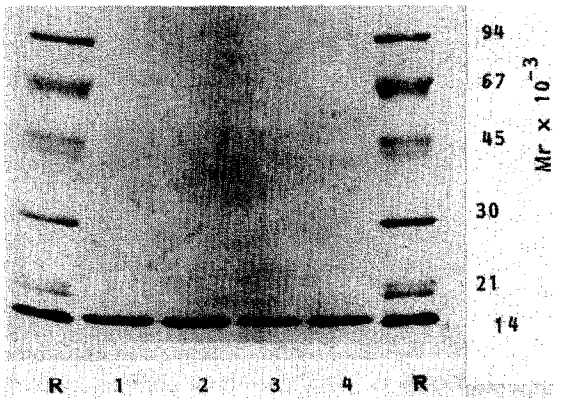

Fig.1. SDS-PAGE of RSA in the presence (lanes 2–4) and absence (lane 1) of mercaptoethanol. Purified RSA (20 μg) was loaded on lanes 1 and 2; lanes 3 and 4 were loaded with RSA desorbed from the gum arabic Sepharose with 0.1 M galactose (in PBS) and 20 mM unbuffered 1,3 diaminopropane. Lanes R contain M_r marker proteins (lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase *b*).

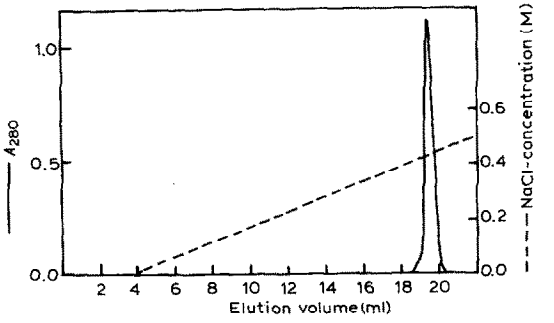

Fig.2. Ion-exchange chromatography of RSA. 5 mg of affinity-purified RSA was chromatographed on a Mono-S column (type HR 5/5 from Pharmacia) using a Pharmacia FPLC system. After washing the column with 4 ml of formate buffer, the lectin was eluted with a linear gradient (30 ml) of increasing NaCl concentration (from 0 to 0.6 M) in the same buffer. The flow rate was 2 ml/min. The A_{280} of the eluate was continuously recorded. Fractions (0.5 ml) were collected and their agglutination determined with trypsin-treated rabbit erythrocytes.

about 150 mg lectin per 1 culture. Moreover, the relative lectin concentration of the mycelium is high since, as shown in table 1, about 11% of the total mycelial protein is RSA.

3.2. *Molecular structure and biochemical properties*

Both reduced (with 2% β-mercaptoethanol) and unreduced RSA migrated as a single polypeptide

Table 1

Purification of RSA

Purification step	Volume (ml)	Total protein (mg)	Total aggl. activity (aggl. units)[b]	Specific aggl. activity (aggl. units/mg protein)[b]	Yield (%)
Crude extract[a]	100	193	120000	622	100
Affinity chromatography	25	23.2	116000	5000	96.6
Ion-exchange chromatography	12	21.8	110000	5046	91.6

[a] 2 g powdered dry mycelium (corresponding to 200 ml of a liquid culture of *R. solani*) was used

[b] An agglutinating unit is defined as the amount of lectin required for the agglutination of 1 ml of a 1% suspension of trypsin-treated rabbit erythrocytes (which correspond to an absolute amount of about 0.20 μg pure RSA)

band upon SDS-PAGE with an apparent M_r of 13000 (fig.1). Since the native lectin was eluted from a Superose 12 gel filtration column with an apparent M_r of 25000 (fig.3), RSA is a dimeric protein composed of 2 identical subunits. Both in the presence of 0.1 M galactose (fig.3) and in its absence (not shown) the lectin was eluted at exactly the same position from the Superose 12 column. Amino acid analysis indicated a high content of asparagine/aspartic acid, valine, glycine, glutamine/glutamic acid and lysine; no sulfur amino acids could be detected (table 2). The lectin does not contain measurable amounts of either amino sugars or neutral sugars, which shows that it is not glycosylated.

Purified RSA is stable within a pH interval between 4.0 and 11.0. At lower and higher pH values, however, its agglutination activity rapidly decreases. The lectin withstands heating at 50°C for 10 min; at higher temperatures, however, it becomes rapidly inactivated.

3.3. *Agglutinating properties and carbohydrate-binding specificity*

RSA agglutinates both human and rabbit erythrocytes. However, as shown in table 3, the lectin has a much higher activity with the latter. With respect to human red blood cells, RSA exhibits a clear preference for type A over type B and O erythrocytes. Trypsin treatment increases the sensitivity of both rabbit and human red blood cells but clearly to a different extent.

The carbohydrate-binding specificity of RSA was determined in hapten inhibition assays with a series of simple sugars and some commonly used glycoproteins (table 4). *N*-Acetylgalactosamine was by far the most potent inhibitor, being 6-times more inhibitory than galactose or α-linked galactose di- or trisaccharides (melibiose or raffinose), 30-times more inhibitory than β-linked galactose (lactose) or 6-deoxygalactose (D-fucose), 45-times more inhibitory than galactosamine and 90-times more inhibitory than L-arabinose (the pentose which has the same configuration of the ring hydroxyls as D-galactose). Ribose and *meso*-erythritol were only slightly inhibitory but more potent inhibitors than arabinitol, xylose and mannose. All other sugars tested (including L-fucose) were not inhibitory at concentrations below

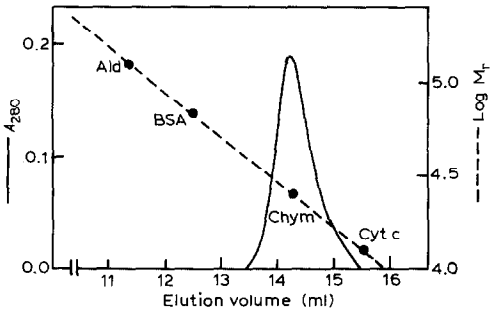

Fig.3. Gel filtration of RSA. 1 mg of purified RSA was chromatographed on a Superose 12 column (type HR 10/30 from Pharmacia) using a Pharmacia FPLC system in PBS containing 0.1 M galactose (to prevent possible binding of the lectin to the column). Flow rate was 20 ml/h. The A_{280} was continuously recorded. Fractions (0.2 ml) were collected and their agglutination titer determined with trypsin-treated rabbit erythrocytes. Elution positions of M_r marker proteins are indicated: aldolase (Ald, M_r 160000); bovine serum albumin (BSA, M_r 67000); chymotrypsinogen (Chy, M_r 23000); cytochrome *c* (Cyt c, M_r 12500).

Table 2

Amino acid composition of RSA

Amino acid	mol%	Residues/mol (nearest integer)
AsX	12.6	15
Thr	8.8	11
Ser	8.2	10
GlX	9.3	11
Pro	3.2	4
Gly	9.7	12
Ala	7.3	9
Cys	0.0	0
Val	10.3	12
Met	0.0	0
Ile	2.3	3
Leu	7.6	9
Tyr	3.6	4
Phe	2.1	3
His	1.4	2
Lys	8.1	10
Trp	3.2	4
Arg	2.3	3
Total per	100	122
Calculated M_r		13243

Table 3

Agglutinating activity of purified RSA in assays with rabbit and human erythrocytes

Erythrocytes from	Minimal concentration (μg/ml) of RSA required for agglutination	
	Untreated	Trypsin-treated
Rabbits	0.8	0.2
Humans		
type A	60	1.5
type B	240	4
type O	500	20

Table 4

Carbohydrate-binding specificity of RSA

Sugar	Concentration (mM)[a]
N-Acetylgalactosamine	0.1
Melibiose	0.6
Galactose	0.6
Raffinose	0.6
Lactose	3.0
D-Fucose	3.0
Galactosamine	4.5
Rhamnose	6.0
Arabinose	9.0
Ribose	18.0
Mesoerythritol	37.5
Arabitol	50.0
Xylose	50.0
Mannose	75.0
Xylitol	100.0
Gentiobiose	100.0
Arabic gum	0.5 mg/ml

[a] Concentration required for 50% inhibition of the agglutination of trypsin-treated rabbit erythrocytes in the presence of 1 μg/ml RSA

Other sugars which were tested (L-fucose, glucose, cellobiose, maltose, fructose, sucrose, trehalose, glucosamine, *N*-acetylglucosamine, glucuronic acid, mannosamine, galacturonic acid, L-sorbose, sorbitol) were not inhibitory; also the glycoproteins fetuin, asialofetuin, ovomucoid and thyroglobulin, had no inhibitory effect at concentrations below 5 mg/ml 100 mM. Similarly, none of the glycoproteins we used had any inhibitory effect at least at concentrations below 5 mg/ml. The (hetero) polysaccharide gum arabic (which contains galactose, arabinose and rhamnose), however, was a reasonably potent inhibitor, being effective at 0.5 mg/ml.

4. DISCUSSION

The mycelium of *R. solani* grown in liquid medium contains relatively large amounts of a soluble intracellular agglutinin, which can readily be isolated by affinity chromatography on immobilized gum arabic. The purified lectin is a small dimeric protein composed of 2 identical subunits (M_r 13000) which is unusually rich in lysine but completely devoid of cysteine, methionine and covalently bound carbohydrate. In hapten inhibition assays, RSA clearly behaves as an *N*-acetylgalactosamine specific lectin, which, however, is also inhibited by several other simple sugars, including D-fucose but not L-fucose. The most important features which favour binding seem to be that the substituents at carbons 2 and 4 should be in the same orientation as in galactose. Based on the observation that D-fucose is a reasonably potent inhibitor of RSA whereas L-fucose is completely inactive, and the fact that RSA is a soluble mycelial protein, it can be concluded that the lectin we isolated must be a different protein from the L-fucose binding agglutinin associated with the cell wall of *R. solani* [11].

At present there are no indications for the physiological role RSA fulfills. However, its relative abundance in the mycelium suggests that it is an important protein in this particular stage of the life cycle of the fungus.

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